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We recently identified a novel transcription factor, LMO-4, which exhibits prominent expression in epithelial cells, including that of the breast. Since previous members of the LIM only (LMO) gene family are oncogenes in lymphocytes, we hypothesized that LMO-4 may play a role in mammary gland development and cancer. To test this prediction, we are in the process of 1. Defining more precisely the expression of LMO-4 during mammary gland development and in human breast cancer, 2. Analyzing transgenic mice which we have created to alter the activity of LMO-4 in mammary glands, and 3. Cloning DNA-binding protein with which LMO-4 interacts. We have found that LMO-4 is primarily expressed in the mammary gland during a stage when epithelial cells are proliferating in preparation for lactation. In addition, est databases show that LMO-4 is highly expressed in human breast cancer. We have generated transgenic lines for analyses and initiated cloning of LMO-4-interacting proteins. Our preliminary data suggest that expression of LMO-4 is associated with undifferentiated cellular stage, such as that found in breast cancer.

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INTRODUCTION:

Understanding the mechanisms involved in regulating proliferation and differentiation of breast epithelial cells is important for further understanding the causes, diagnosis and treatment of breast cancer. We have recently identified a new protein called LMO-4, a member of a family of proteins that participate in gene regulation. Proteins belonging to this group have been shown to cause leukemia. We were therefore intrigued to find that LMO-4 is highly abundant in breast epithelial cells when these cells are proliferating. Our hypothesis is therefore that LMO-4 is involved in regulation of proliferation of breast epithelial cells in normal breast and in breast cancer. To test this hypothesis, we are pursing the following **Specific Aims**:

#1. Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.

#2. Define the role of LMO-4 in normal breast development and in breast cancer, using a mouse transgenic approach.

#3. Identify and characterize protein partners for LMO-4 in human breast tissue.

BODY:

Objective #1. Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.

a. Raise and purify LMO-4 antisera.

For the expression studies, we have elected to use immunohistochemistry and *in situ* hybridization as complementary techniques. Our primary approach will be immunohistochemistry, as direct detection of protein expression is critical given that mRNA levels do not always correlate with protein levels. Additionally, immunolocalization is more precise, permits subcellular localization, and since proteins are less sensitive to degradation than mRNA, provides an advantage when using tissue specimens from patients. Consequently, we have focused on creating good LMO-4 antisera. Progress in the beginning was somewhat slow because the protein proved insoluble, but after adapting a different bacterial expression and purification strategy, we were able to express large amounts of highly purified LMO-4 protein.

The protein was injected both into rabbits (2 animals) and guinea pigs (3 animals). We have just started to characterize the antisera by immunohistochemistry. For testing, we have selected mouse embryos since these express LMO-4 at a high level in several locations. In preliminary experiments we have had trouble obtaining consistent immunostaining on paraffin embedded tissue. We will continue to work out conditions for immunostaining by altering fixation and also testing fresh frozen tissue. If we do not succeed in using these antibodies, we will fall back on using in situ hybridization.

b. Obtain mouse embryos and pregnant mice at different developmental stages.

We have obtained fixed embedded mammary glands from mice at different developmental stages for use in immunohistochemistry and *in situ* hybridization studies. We are also in the process of isolating RNA from mammary glands at different developmental stages for studying LMO-4 mRNA levels with RNAse protection assays. During the coming year we plant to initiate immunohistochemistry studies with human breast tumors in an effort to determine the pattern and level of LMO-4 expression in breast tumors. Expression of LMO-4 in breast cancer cells will be correlated with tumor type, the degree of tubule formation, degree of nuclear pleomorphism and number of mitotic counts.

c. Study LMO-4 expression during normal mammary gland development.

Our initial in situ hybridization studies indicate that LMO-4 levels in mammary glands are highest during midpregnancy and become undetectable after weaning. Together, these experiments in mice and human breast cancer will help us to correlate the expression of LMO-4 with proliferation and differentiation status of breast epithelial cells, whereby we will be able to form more precise hypotheses as to the role of LMO-4 in normal and neoplastic breast.

Ojective #2. **Define the role of LMO-4 in normal breast development and in breast cancer, using a transgenic approach.**

Two previously characterized members of the LMO-family, LMO-1 and LMO-2, have been found to be oncogenic. In humans these genes are overexpressed in lymphocytes due to fusion with the T-cell receptor in chromosomal translocations associated with acute lymphoblastic leukemia. These observations suggest that the LMO class of proteins plays roles in regulation of both proliferation and differentiation critical for organ development and that abnormalities in LMO-activity may lead to oncogenesis. Our hypotheses is that LMO-4 plays a role in normal breast development and that subversion of LMO-4 function or activity may contribute to formation of breast tumors.

We have elected to test our hypothesis using a transgenic approach, which allows us to test the role of LMO-4 in the context of the whole animal. We have made significant progress towards this goal. We have decided to create four sets of transgenic mice: one in which LMO-4 is overexpressed, one in which LMO-4 is converted into a "superactivator", one in which LMO-4 activity is inhibited, and a fourth one in which we have overexpressed a dominant negative form of the LMO-4-associated protein, CLIM.

a/b. Creation and testing of transgenic plasmids and microinjection of oocytes for establishing transgenic lines.

Overexpression of LMO-4: The LMO-4 cDNA fused in frame with a MYC epitope is expressed under the control of the mouse mammary tumor virus (MMTV) enhancer/promoter. This plasmid is under construction but has not been injected into oocytes.

Overexpression of LMO-4/VP-16 activation domain fusion: The HA-tagged LMO-4 cDNA was fused in frame with the activation domain of VP-16 and placed under the control of the MMTV promoter. This fusion molecule should create an LOM-4 superagonist. This construct has already been injected into oocytes and out of 53 pubs we have 12 transgenic founder mice.

Overexpression of LMO-4/engrailed repression domain fusion: The HA-tagged LMO-4 cDNA was fused in frame with the repression domain of engrailed and placed under the control of the MMTV promoter. The engrailed repression domain is dominant and overcomes transcriptional activation. Thus, we will create an artificial repressor that maintains the protein-protein interaction specificity of LMO-4. LMOs are thought to mediate their action by associating with DNA-binding proteins and simultaneously serving as docking proteins for the co-activator proteins CLIMs/Nli/Ldb that are thought to participate in transcriptional activation. The goal of our experiment is to overexpress the LMO-4/engrailed fusion to introduce a strong repressor domain into these DNA-protein complexes that will interfere with transactivation and instead repress LMO-4 target genes. This construct has already been injected into oocytes and out of 13 pubs we have 5 transgenic founder mice.

Overexpression of a dominant negative CLIM molecule: The MYC tagged C-terminus of CLIM, which interacts with LIM domains, was placed under the control of the MMTV promoter. This approach has been previously used to interfere with the action of LIM factors. This construct has already been injected into oocytes and out of 57 pubs we have 4 transgenic founder mice.

Our next step is to confirm that the transgenes are expressed in mammary epithelial cells, using immunohistochemistry (antibody against the HA- and MYC tags). Transgenic mice are being bred to C57BL/6 mice, which have low incidence of mammary cancer, to allow generation of sufficient female mice for analyses. So far, all transgenic mothers seem to be able to lactate.

Objective #3. **Identify and characterize protein partners for LMO-4 in human breast tissue.**

While LMO and LIM homeobox proteins are similar in that they are both localized to the nucleus, there is no evidence to suggest that the biological activity of LMOs is through direct DNA-binding. Insight into the biochemical mechanisms of actions for LMO proteins came from studies of LMO-1 and -2 in the hematopoietic system where it was found that LMO-2 interacts strongly with the bHLH domain of TAL1 and that these proteins, as well as Clim-2, exist in a complex in erythroid cells. These experiments suggest a model in which LMO factors can be tethered to DNA by associating with DNA binding proteins, thus allowing the co-regulator CLIM to interact with transactivators that do not contain a covalently linked LIM domain.

We therefore propose that a LMO-4 and Clim-2 containing complex regulates gene activity in breast epithelial cells by associating with unidentified DNA-binding protein(s). The goal of the proposed experiments is to identify such factor(s). Specifically, we are interested in determining whether LMO-4 may interact with transcription factors or nuclear oncoproteins that have been shown to regulate differentiation and proliferation in normal and neoplastic breast.

a/b. Construction of yeast two hybrid libraries and screening with LMO-4 bait.

We are currently obtaining RNA for construction of mouse yeast two-hybrid library. However, we already have access to a human breast library. In an effort to isolate LMO-4-interacting proteins directly, we have used the yeast two-hybrid screening system. In this approach, LMO-4 fused with the GAL4 DNA-binding domain (bait) was introduced into a yeast strain containing GAL4 DNA-binding sites regulating the *HIS3* and *lacZ* genes. To identify LMO-4-interacting proteins, DNA from a human breast HybriZAP library where cDNAs are fused to the GAL4 activation domain (target) was introduced with the LMO-4 bait into the yeast reporter strain. Since the growth media lacks histidine, yeast will only grow if there is an interaction between LMO-4 and a target protein expressed from a library cDNA leading to activation of the *HIS3* gene. Interacting clones will also exhibit beta-galactosidase activity.

In a pilot screen of a normal human breast library, we isolated both CLIM-2 and human DEAF-1. Recently, we have completed another round of screening and found 9 clones that show bait-dependent activation. By restriction analyses, at least 5 of these clones appear to be distinct from DEAF-1 and CLIM-2. Further analyses has showed that one of these clones is a DNA-binding factor, Zn43 (1). Intriguingly, two of the factors isolated in this screen are components of the splicosome, protein M4 and SPF27 (2). So far LMO factors have not been implicated in regulation of splicing, but recent data suggest that transcription factors may play a role in regulation of splicing. In addition, it is highly interesting that the gene expressing one of these factors, SPF27, was found to be highly amplified in human breast carcinoma cell lines. This gene has also been referred to as DAM1 (DNA amplified in mammary carcinoma) because it was isolated in screens designed to identify transcripts upregulated in human carcinoma cell lines (3).

KEY RESEARCH ACCOMPLISHMENTS:

1. Creation of LMO-4 antisera.
2. Establishment of a "bank" of mammary glands from different developmental stages, suitable for expression analyses.
3. Preliminary outlining of LMO-4 expression during development.
4. Establishment of high LMO-4 expression in human breast tumors by analyses of est gene databases.
5. Creation of transgenic constructs.
6. Establishment of transgenic lines in which LMO-4 has been selectively perturbed in mammary gands of mice.
7. Identification of LMO-4-interacting proteins in yeast two hybrid screen from human breast library.

REPORTABLE OUTCOMES:

1. Development of antisera
2. Transgenic mouse models for LMO expression
3. A fellowship award (BC000553) was funded based on work on this project.

CONCLUSIONS:

In summary, we have made significant progress on all three specific aims. A great deal of groundwork has been completed in the first year of the award, which include the creation of key reagents such as antisera, tissue banks and transgenic founder lines. Our early results suggest that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found in breast cancer. With our work, we hope to gain new insights into the causes of breast cancer and to define genes and proteins that could potentially lead to new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer

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